National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2396

Oxidative DNA Damage
Mass Spectrometry Standards

This Standard Reference Material (SRM) is intended for use in the measurement of oxidative DNA damage by gas chromatography/mass spectrometry (GC/MS) [1], and liquid chromatography/mass spectrometry (LC/MS) [2], using the isotope-dilution technique for quantification in both cases. SRM 2396 is a set of twelve stable isotope-labeled components (ten analogues of oxidatively modified DNA bases, one analog of an oxidatively modified nucleoside and one analog of a normal DNA nucleoside) contained in a protective plastic box (Table 1). Each vial of SRM 2396 contains 0.2 mL of a designated component at a specified concentration listed in Table 2.

The compounds contained in this SRM are the stable isotope-labeled analogues of some major modified bases (components 1 through 10) and a modified nucleoside (component 12) that result from oxidative damage to DNA. Included is also one normal DNA nucleoside (component 11). The structures of these compounds are illustrated in Figure 1. The mechanisms, by which these products are formed, have been studied extensively and elucidated in the past [2].

Certified Values: The certified concentration value for each component is provided in Table 2.

Expiration of Certification: The certification of this SRM is valid until **01 March 2014**, provided the SRM is handled and stored in accordance with the instructions given in this certificate. This certification is nullified if the SRM is damaged, contaminated, or modified.

Maintenance of SRM Certification: NIST will monitor this SRM over the period of its certification. If substantive technical changes occur that affect the certification before the expiration of this certificate, NIST will notify the purchaser. Registration (see attached sheet) will facilitate notification.

The overall direction and coordination of the technical measurements leading to the certification were performed by H. Rodriguez and M. Dizdaroglu of the NIST Biotechnology Division.

The analytical determination, technical measurements and analysis of data for the certification of this SRM were performed by H. Rodriguez, P. Jaruga and M. Dizdaroglu of the NIST Biotechnology Division.

The support aspects involved in the issuance of this SRM were coordinated through the NIST Standard Reference Materials Program by C.S. Davis of the NIST Measurement Services Division.

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Table 1. Nomenclature of the Certified Components

Component ID	Name
1	4,6-Diamino-5-formamidopyrimidine-(formyl-^{13}C-4,6-diamino-$^{15}N_2$): abbreviated on the tube as FapyAde-^{13}C,$^{15}N_2$
2	2,6-Diamino-4-hydroxy-5-formamidopyrimidine-(formyl-^{13}C-5-amido-6-amino-$^{15}N_2$): abbreviated on the tube as FapyGua-^{13}C,$^{15}N_2$
3	8-Hydroxyadenine-8-^{13}C-9-^{15}N-(6-diamino-^{15}N): abbreviated on the tube as 8-OH-Ade-^{13}C,$^{15}N_2$
4	5-Hydroxycytosine-2-^{13}C-1,3-$^{15}N_2$: abbreviated on the tube as 5-OH-Cyt-^{13}C,$^{15}N_2$
5	5-Hydroxyuracil-2,4,5,6-$^{13}C_4$-1,3-$^{15}N_2$: abbreviated on the tube as 5-OH-Ura-$^{13}C_4$,$^{15}N_2$
6	5-(Hydroxymethyl)uracil-4,5-$^{13}C_2$-α,α-$^{2}H_2$: abbreviated on the tube as 5-(OHMe)Ura-$^{13}C_2$,d_2
7	*cis*-Thymine glycol-α,α,α,6-$^{2}H_4$: abbreviated on the tube as ThyGly-d_4
8	5-Hydroxy-5-methylhydantoin-2-^{13}C-1,3-$^{15}N_2$: abbreviated on the tube as 5-OH-5-MeHyd-^{13}C,$^{15}N_2$
9	Isodialuric acid-2-^{13}C-1,3-$^{15}N_2$: abbreviated on the tube as isodialuric acid-^{13}C-$^{15}N_2$ (also known as its enol form 5,6-dihydroxyuracil-^{13}C-$^{15}N_2$)
10	Dialuric acid-1,3-$^{13}C_2$-2,4-$^{15}N_2$: abbreviated on the tube as dialuric acid-$^{13}C_2$-$^{15}N_2$ (this compound is spontaneously oxidized in aqueous solution, and quantitatively converted into alloxan-$^{13}C_2$-$^{15}N_2$ [1,3]).
11	2′-Deoxyguanosine-$^{15}N_5$: abbreviated on the tube as dGuo-$^{15}N_5$
12	7,8-Dihydro-8-oxo-2′-deoxyguanosine-$^{15}N_5$ (known as 8-hydroxy-2′-deoxyguanosine-$^{15}N_5$): abbreviated on the tube as 8-OH-dGuo-$^{15}N_5$

Components 1 through 10 are used as internal standards for the measurement of the corresponding unlabeled analogues in DNA by GC/MS [1]. Component 11 quantitatively yields Gua-$^{15}N_5$ upon acidic hydrolysis and is used as an internal standard for the measurement of Gua in DNA by GC/MS, thus the amount of DNA [4]. Component 12 is used as an internal standard for the measurement of 8-OH-dGuo in DNA by LC/MS after enzymic hydrolysis of DNA [5], and for the measurement of 8-OH-Gua in DNA by GC/MS after acidic hydrolysis of DNA [6].

Table 2. Certified Concentrations[a] and Uncertainties of the Components

Component ID	Compound	Concentration (mM)	*k*
1	FapyAde-$^{13}C,^{15}N_2$	0.2290 ± 0.000989	2.2
2	FapyGua-$^{13}C,^{15}N_2$	0.1556 ± 0.003666	2.2
3	8-OH-Ade-$^{13}C,^{15}N_2$	0.1440 ± 0.000473	2.2
4	5-OH-Cyt-$^{13}C,^{15}N_2$	0.2267 ± 0.000334	2.2
5	5-OH-Ura-$^{13}C_4,^{15}N_2$	0.1280 ± 0.000414	2.2
6	5-(OHMe)Ura-$^{13}C_2, d_2$	0.0978 ± 0.002408	2.2
7	ThyGly-d_4	0.1039 ± 0.002317	2.2
8	5-OH-5-MeHyd-$^{13}C,^{15}N_2$	0.0933 ± 0.001362	2.2
9	Isodialuric Acid-$^{13}C,^{15}N_2$	0.1207 ± 0.002188	2.2
10	Dialuric Acid-$^{13}C_2,^{15}N_2$	0.0981 ± 0.002627	2.2
11	dGuo-$^{15}N_5$	0.1148 ± 0.001194	2.2
12	8-OH-dGuo-$^{15}N_5$	0.0881 ± 0.001252	2.2

[a] The certified values are the means of the measurement results obtained by NIST by way of methods previously described. The expanded uncertainty displayed is calculated as $U = ks$, in accordance to the NIST and ISO Guides [19], where s represents one standard deviation of the average of the 12 measurements made on each component. The coverage factor, k, is used to obtain an expanded uncertainty with the desired confidence level. The value of the coverage factor is determined from the Student's t-distribution with 11 degrees of freedom and a confidence level of 95 %.

NOTICE AND WARNING TO USER

WARNING: THIS PRODUCT IS NOT RADIOACTIVE. HEALTH AND SAFETY DATA FOR THE LABELED COMPOUNDS ARE GENERALLY UNAVAILABLE, BUT ARE ASSUMED TO BE SIMILAR OR IDENTICAL TO THE CORRESPONDING UNLABELED COMPOUND. THE PRODUCT IS TO BE HANDLED AS A BIOHAZARDOUS CHEMICAL MATERIAL.

Storage: Store frozen at a temperature of –20 °C. **DO NOT** use a self-defrosting freezer because the periodic cycling of temperatures may shorten the shelf life of this SRM.

INSTRUCTIONS FOR USE

It is recommended that once thawed, each SRM component should be used in its entirety. Repeated freezing and thawing is **NOT** recommended as this might shorten the shelf life of the SRM. If it is necessary to perform repeated analyses, thaw the SRM and divide the tube contents into aliquots that will be kept frozen until use. Thawing can be conducted at refrigerator temperatures or room temperature. Once thawed, the sample should be processed without delay.

SOURCE AND ANALYSIS[1]

Source of Material: Components 1 through 8, and 11 and 12 were purchased from Cambridge Isotope Laboratories (Andover, MA). Components 9 and 10 were custom-synthesized by Program Resources Inc./Dyncorp, National Cancer Institute (Frederick, MD) [7].

NIST Analyses: Measurements were carried out in the DNA Damage & Repair research laboratory, while packaging of the components was carried out in a laminar flow hood. The laminar flow hood was sterilized prior to packaging by washing the hood with 70 % ethanol, wiping the work area with germicidal disposable cloths, and running the UV sterilization light of the laminar flow hood for a period of twelve hours. The vials were sterile when received and remained sealed prior to use. The vials were processed in racks of 100 vials. Each tube was checked for tightness of the seal prior to storage at –20 °C.

Each component was received in solid state and stored at –20 °C prior to preparation. The compounds were weighed in an analytical chemistry weighing room at a relative humidity of 47.5 %. Using an analytical microbalance and the molecular weights listed in Table 3, an appropriate amount of each compound was weighed separately and dissolved in a required amount of HPLC-grade water.

The concentration of the solution of each compound was determined by either GC/MS or LC/MS. In the case of UV light-absorbing compounds, UV-spectroscopy was also used to confirm the GC/MS and LC/MS measurements. Table 3 shows the techniques used for determination of the concentration of each component. A total of 12 independent measurements for each labeled component and each corresponding non-labeled analog were performed on three separate days for statistical purposes, as recommended by a NIST statistician.

GC/MS: The concentrations of the components 1 through 10 were measured by GC/MS. The corresponding non-labeled compounds were used as internal standards. Non-labeled compounds were custom-synthesized by the Program Resources Inc./Dyncorp, National Cancer Institute (Frederick, MD) [7], except for FapyAde and 5-(OHMe)Ura, which were purchased from Sigma Chemical Company (St. Louis, MO). The concentrations of the solutions of these compounds were determined by weight and by UV-spectroscopy. A 0.1 mL aliquot of the solution of a compound was used for the measurement. Absorption spectra of the solutions were recorded between 210 nm and 350 nm. Using the known absorption coefficient of each compound and the absorption at the recorded absorption maximum, the concentrations of the compounds in solution were calculated. The absorption coefficient and absorption maximum of each compound are given in Table 4. The concentrations of the compounds, ThyGly-d_4; 5-OH-5-MeHyd-^{13}C,$^{15}N_2$; isodialuric acid-^{13}C-$^{15}N_2$ and dialuric acid-$^{13}C_2$-$^{15}N_2$, which do not absorb

[1]Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedures. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

UV-light in the designated wavelength range, were determined by GC/MS using the corresponding non-labeled compounds as internal standards. The concentrations of the solutions of these four compounds were based on weight. Non-labeled alloxan was also used to determine the concentration of the solution of dialuric acid-$^{13}C_2$-$^{15}N_2$, since dialuric acid is oxidized and quantitatively converted into alloxan in aqueous solution [1,3]. Upon acidic hydrolysis, alloxan-$^{13}C_2$-$^{15}N_2$ quantitatively yields 5-hydroxyhydantoin-$^{13}C_2$-$^{15}N_2$ (5-OH-Hyd-$^{13}C_2$-$^{15}N_2$) [8].

GC/MS with electron-impact (EI) ionization was performed using a gas chromatograph-mass selective detector. The column was a fused silica capillary column (12.5 m × 0.2 mm i.d.) coated with cross-linked 5 % phenylmethylsilicone gum phase (film thickness, 0.33 μm). Aliquots of labeled and non-labeled compounds were mixed and the mixtures were lyophilized in glass vials to dryness for 18 h. For derivatization of dried samples, 60 μL of a mixture of nitrogen-bubbled bis(trimethylsilyl)trifluoroacetic acid (BSTFA) (containing 1 % trimethylchlorosilane) and pyridine (1:1, v/v) were added to the vials. The vials were purged individually with ultra high-purity nitrogen, vortexed and then tightly sealed under nitrogen with Teflon-coated septa. The derivatization was carried out at 120 °C for 30 minutes. Aliquots of 0.002 mL of the derivatized samples were injected onto the GC-column by means of an automatic sampler. The split mode of injection with a split ratio of 10 to 1 was used. The selected-ion monitoring mode was used to monitor the characteristic ions of the trimethylsilyl (TMS) derivatives of the components 1 through 10 in their EI mass spectra [1,8-11]. These mass spectra are dominated by a molecular ion ($M^{+\bullet}$) and an $(M-15)^+$ ion, which results from loss of a methyl radical from $M^{+\bullet}$. In some instances, loss of an H atom from $M^{+\bullet}$ gives rise to an $(M-1)^+$ ion. Some fragment ions are also formed. Upon acidic hydrolysis, dGuo-$^{15}N_5$ quantitatively yields Gua-$^{15}N_5$, which is used as an internal standard to determine the content of Gua in DNA, and thus the amount of DNA in DNA samples [4]. Similarly, 8-OH-dGuo-$^{15}N_5$ yields 8-OH-Gua-$^{15}N_5$, which is used as an internal standard to measure the content of 8-OH-Gua in DNA after acidic hydrolysis. Table 5 shows the masses of the characteristic ions of the TMS derivatives of the components 1 through 12 and their unlabeled analogues that can be used for identification and quantification.

LC/MS: The concentrations of the components 11 and 12 were determined by LC/MS with the atmospheric pressure ionization-electrospray (API-ES) process in the positive ionization mode, using recently developed methodologies [5] and corresponding non-labeled analogues as internal standards. The concentrations of their solutions were determined by weight and by UV-spectroscopy, using the known absorption coefficient of each compound and the absorption at the recorded absorption maximum (Table 4). LC/MS analyses were carried out using a liquid chromatograph-mass selective detector. Separations were performed using a ZORBAX Eclipse XDB C18-reversed-phase column (15 cm × 0.21 cm i.d., 5 μm particle size) (Agilent Technologies) with a guard column packed with the same stationary phase (1 cm × 0.21 cm i.d.). The mobile phase used was a gradient of 0.5 % of solvent B/min where solvent A was a mixture of water and acetonitrile (98/2, v/v) and the solvent B was acetonitrile. A gradient of 0.5 % of the solvent B/min was used. The flow rate was 0.2 mL/min. The column temperature was kept at 30 °C [5,12]. The selected-ion monitoring mode was used to monitor the characteristic ions of the API-ES mass spectra of the components 11 and 12. These mass spectra consist of a protonated molecular ion (MH^+), a base ion with two H atoms (BH_2^+), and a sodium adduct ion (MNa^+). The masses of the ions of dGuo-$^{15}N_5$ are 273 (268) Da, 157 (152) Da and 295 (290) Da, respectively, whereas those of 8-OH-dGuo-$^{15}N_5$ amount to 289 (284) Da, 173 (168) Da and 311 (306) Da, respectively. The numbers in parentheses are the masses of the ions of the unlabeled analogues [5,13].

SRM Final Assembly: Final concentrations of the components are listed in Table 2. The sterile microcentrifuge tubes with attached screw caps were pre-labeled. Working inside a laminar flow hood, single component tubes were placed into bleached microtube racks (96-well format) and the caps removed. The solutions were dispensed as 0.2 mL aliquots into the tubes and their lids tightened. The capped filled tubes were placed in Styrofoam microtube racks for –20 °C storage. The units of 12 components were assembled in one batch. One hundred vials of each component were removed from storage. The vials were assembled as quickly as possible in sequential order in pre-labeled plastic SRM boxes. The assembled units were stored at –20 °C.

Table 3. Components of SRM 2396, Molecular Weights, Solution Preparation Date, and the Methods Used to Determine the Concentration

Component ID	Compound	Molecular Weight	Date Solution Prepared	Technique Used to Determine Concentration	
				GC/MS	LC/MS
1	FapyAde-$^{13}C,^{15}N_2$	156	8-May-2003	X	
2	FapyGua-$^{13}C,^{15}N_2$	172	8-May-2003	X	
3	8-OH-Ade-$^{13}C,^{15}N_2$	154	8-May-2003	X	
4	5-OH-Cyt-$^{13}C,^{15}N_2$	130	8-May-2003	X	
5	5-OH-Ura-$^{13}C_4,^{15}N_2$	134	8-May-2003	X	
6	5-(OHMe)Ura-$^{13}C_2, d_2$	146	9-May-2003	X	
7	ThyGly-d_4	164	9-May-2003	X	
8	5-OH-5-MeHyd-$^{13}C,^{15}N_2$	133	9-May-2003	X	
9	Isodialuric Acid-$^{13}C,^{15}N_2$	147	23-June-2003	X	
10	Dialuric Acid-$^{13}C_2,^{15}N_2$	148	23-June-2003	X	
11	dGuo-$^{15}N_5$	272	8-July-2003		X
12	8-OH-dGuo-$^{15}N_5$	288	15-June-2004		X

Table 4. Wavelengths at the Maximum Absorption of the Components and the Absorption Coefficients

Component ID	Compound	Wavelength (nm)	Absorption coefficient ($L\ mol^{-1}\ cm^{-1}$)
1	FapyAde-$^{13}C,^{15}N_2$	262	4710 [14]
2	FapyGua-$^{13}C,^{15}N_2$	266	14932 [14]
3	8-OH-Ade-$^{13}C,^{15}N_2$	268	12764 [14]
4	5-OH-Cyt-$^{13}C,^{15}N_2$	284	5400[a]
5	5-OH-Ura-$^{13}C_4,^{15}N_2$	278	8200 [15]
6	5-(OHMe)Ura-$^{13}C_2, d_2$	261	8100 [16]
7	ThyGly-d_4	N/A	N/A
8	5-OH-5-MeHyd-$^{13}C,^{15}N_2$	N/A	N/A
9	Isodialuric Acid-$^{13}C,^{15}N_2$	N/A	N/A
10	Dialuric Acid-$^{13}C_2,^{15}N_2$	N/A	N/A
11	dGuo-$^{15}N_5$	254	13000 [16]
12	8-OH-dGuo-$^{15}N_5$	245	12300 [17]
		292	10300 [18]

[a] Determined on the basis of the weight using the unlabeled compound.

Table 5. The Masses (Da) of the Characteristic Ions of the TMS Derivatives of the Components 1 Through 12 and Their Unlabeled Analogues (in Parentheses)[a]

Compound	$M^{+\bullet}$	$(M-1)^+$	$(M-15)^+$	Fragment Ion
FapyAde-$^{13}C,^{15}N_2$	372 (369)	371 (368)	357 (354)	283 (280)
FapyGua-$^{13}C,^{15}N_2$	460 (457)		445 (442)	371 (368)
8-OH-Ade-$^{13}C,^{15}N_2$	370 (367)		355 (352)	
5-OH-Cyt-$^{13}C,^{15}N_2$	346 (343)	345 (342)	331 (328)	
5-OH-Ura-$^{13}C_4,^{15}N_2$	350 (344)	349 (343)	335 (329)	
5-(OHMe)Ura-$^{13}C_2, d_2$	362 (358)		347 (343)	
ThyGly-d_4	452 (448)		437 (433)	262 (259)
5-OH-5-MeHyd-$^{13}C,^{15}N_2$	349 (346)	348 (345)	334 (331)	219 (216)
Isodialuric Acid-$^{13}C,^{15}N_2$[b]	435 (432)		420 (417)	
Dialuric Acid-$^{13}C_2,^{15}N_2$[c] (Alloxan-$^{13}C_2$-$^{15}N_2$)	452 (448)		437(433)	335 (331)
(5-OH-Hyd-$^{13}C_2$-$^{15}N_2$)	336 (332)	335 (331)	321 (317)	
dGuo-$^{15}N_5$[d] (Gua-$^{15}N_5$)	372 (367)		357 (352)	
8-OH-dGuo-$^{15}N_5$[e] (8-OH-Gua-$^{15}N_5$)	460 (455)		445 (440)	

[a] For the details of the mass spectra, see [1,8-11].
[b] Measured as its enol form dihydroxyuracil-$^{13}C,^{15}N_2$ [8].
[c] Measured as alloxan-$^{13}C_2$-$^{15}N_2$ after dissolving in aqueous solution and as 5-OH-Hyd-$^{13}C_2$-$^{15}N_2$ after acidic hydrolysis of DNA samples [8].
[d] Measured as Gua-$^{15}N_5$ after acidic hydrolysis of DNA samples to determine the amount of DNA [4].
[e] Measured as 8-OH-Gua-$^{15}N_5$ to determine the amount of 8-OH-Gua in DNA after acidic hydrolysis [6].

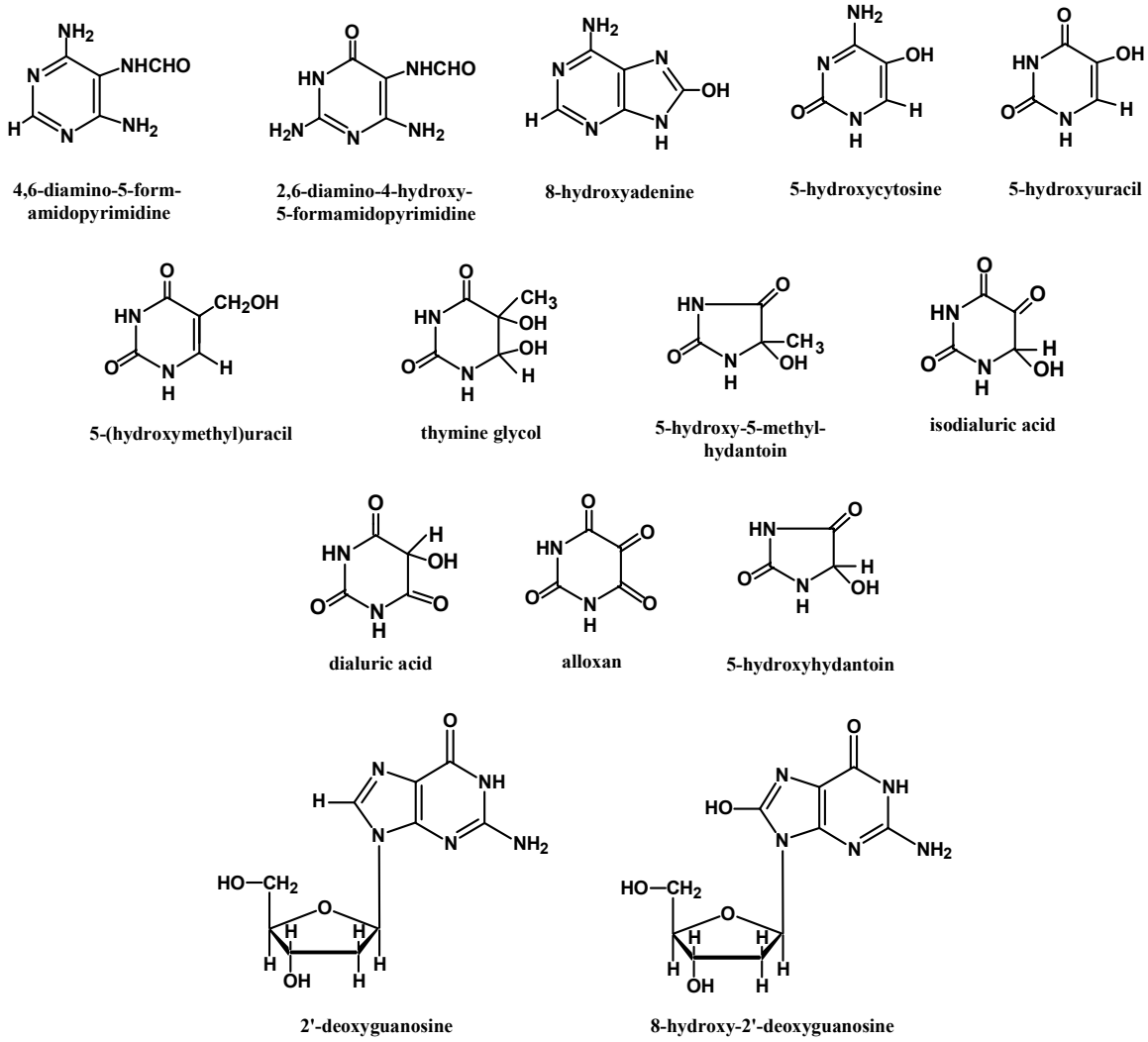

Figure 1. The structures of the components in SRM 2396. Alloxan and 5-hydroxyhydantoin result from oxidation in aqueous solution and acidic treatment of dialuric acid, respectively (see the text).

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Users of this SRM should ensure that the certificate in their possession is current. This can be accomplished by contacting the SRM Program at: telephone (301) 975-6776; fax (301) 926-4751; e-mail srminfo@nist.gov; or via the Internet at http://www.nist.gov/srm.